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(54) Title: LUMINESCENT LANTHANIDE CHELATES AND METHODS OF USE

(57) Abstract

The invention provides lanthanide chelates capable of intense luminescence. The chelates comprise a lanthanide chelator covalently joined to a coumarin-like or quinolone-like sensitizer. Exemplary sensitizers include 2- or 4-quinolones, 2- or 4-coumarins, or derivatives thereof e.g. carbostyril 124 (7-amino-4-methyl-2-quinolone), coumarin 120 (7-amino-4-methyl-2-coumarin), coumarin 124 (7-amino-4-(trifluoromethyl)-2-coumarin), aminomethyltrimethylpsoralen, etc. The chelates form high affinity complexes with lanthanides, such as terbium or europium, through chelator groups, such as DTPA. The chelates may be coupled to a wide variety of compounds to create specific labels, probes, diagnostic and/or therapeutic reagents, etc. The chelates find particular use in resonance energy transfer between chelate-lanthanide complexes and another luminescent agent, often a fluorescent non-metal based resonance energy acceptor. The methods provide useful information about the structure, conformation, relative location and/or interactions of macromolecules.

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Luminescent Lanthanide Chelates and Methods of Use

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INTRODUCTION

Field of the Invention

The field of this invention is luminescent lanthanide chelators.

Background

Luminescent (including fluorescent and phosphorescent) markers find a wide variety of applications in science, medicine and engineering. In many situations, these markers provide competitive replacements for radiolabels, chromogens, radiation-dense dyes, etc. Improvements in fluorimetric instrumentation have increased attainable sensitivities and permitted quantitative analysis.

Perhaps the single-most significant limitation to the use of luminescent markers is generating an acceptable signal-to-noise ratio. Marker-dependent properties such as absorbtion and emission maxima, Stoke's shift, quantum yield, etc. effect the ease of distinguishing signal from auto- or background fluorescence. Hence, there is a continuous need to provide improved luminescent markers;

especially luminescent markers with long-lived luminescence and/or a large Stokes shifts with long wavelenght emmissions. Other useful and desireable properties include: easy and cost-effective synthesis; chemical stability, especially in an aqueous environment; convenient attachability to a wide variety of macromolecules including proteins and nucleic acids; efficient excitability by a convenient laser; capable of intense luminescence; the capacity to perform as good luminescent resonance-energy transfer donors, enabling the determination of molecular distances beyond 100Å; and usefulness as radiation-hardened fluorophores in X-ray microscopy.

Relevant Literature

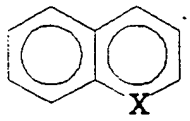
Relevant patents include US Patent Nos 4,637,988 (1987) and 4,837,169 (1989). For a patent application using lanthanide cryptates as energy transfer donors, see U.S. Patent Application, Serial No. 07/729,228, filed July 12, 1991.

DTPA-pAS-Tb is reported in Bailey et al. (1984) *Analyst* 109, 1449-1450. For background papers on other lanthanide chelators, see Diamandis (1992) Analyst 117, 1879-1884, Canfi et al. (1989) Analyst 114, 1405-1406, Ando et al. (1993) Biochimica et Biophysica Acta. 1102, 186-194, Georges and Ghazarián (1993) Analytica Chimica Acta, 276, 401-409, Mathis et al. (1993) *Clin. Chem*, *39*, 1953 and Desai et al. (1993) *J. Am. Chem. Soc.* 115, 11032, Seveus et al. (1992) 13, 329-338, Saavedra and Picozza (1989) Analyst 114, 835-838, von Brenndorff et al. (1993) in Proceedings, 4th Intnl Conf on X-ray Microscopy, Chernogolovoka, Moscow District, Russia, Clark et al. (1993) Analytical Biochemistry 210, 1-6.

For an up-to-date review of Fluorescent Resonance Energy Transfer, see, Selvin (1994) Fluorescence Resonance Energy Transfer, in Biochemical Spectroscopy, a volume of Methods in Enzymology, Academic Press, Ed. Kenneth Sauer, *in press* (copy attached).

SUMMARY OF THE INVENTION

The invention provides lanthanide chelates comprising a lanthanide chelator covalently joined to a polynuclear heterocyclic aromatic sensitizer of the general formula:



where X comprises an atom from periodic group 5 or 6.

In preferred chelates, the sensitizer has a first position 2-8 carbon atom substituted with an oxygen atom through a double covalent bond, a second position 2-8 carbon atom, different than the first position 2-8 carbon atom, substituted with a linking group through which the sensitizer is covalently joined to the chelator, and a substituted third position 2-8 carbon atom, different from the first and second position 2-8 carbon atoms. Frequently, the first position 2-8 carbon atom is the position 2 or 4 carbon atom, the second carbon atom is the position 7 carbon atom, the sensitizer and chelator are linked through an amine or carboxyl group, and/or the third position 2-8 carbon atom is the position 4 carbon and is substituted with a hydrocarbon or halogen substituted hydrocarbon. Exemplary sensitizers include 2- or 4-quinolones, 2- or 4- coumarins, or derivatives thereof e.g. carbostyril 124 (7-amino-4-methyl-2-quinolone), coumarin 120 (7-amino-4-methyl-2-coumarin), coumarin 124 (7-amino-4-(trifluoromethyl)-2-coumarin), aminomethyltrimethylpsoralen, etc.

The chelates are capable of forming high affinity complexes with lanthanides, such as terbium or europium, through chelator groups, such as DTPA. Typically, the chelators comprise a plurality of structurally constrained anionic groups such as carboxylate or phosphonate groups. Solutions of chelate-lanthanide complexes are capable of intense luminescence. The chelates may be coupled to a wide variety of compounds to create specific labels, probes, diagnostic and/or therapeutic reagents, etc.

Chelate-lanthanide complexes are useful as detectable labels in a wide variety of applications. Generally, the methods involve contacting a sample portion with a luminescent complex; exposing the sample portion to light at a first wavelength capable of inducing a first electronic transition in the chelate; and detecting an emission of light from the sample portion at a second wavelength that is longer than the first wavelength and results from a second electronic transition in

the chelate. Specific analytes in the sample may be detected by coupling the chelate to a reagent capable of analyte selectively binding.

The chelates also find use in resonance energy transfer between chelate-lanthanide complexes and another luminescent agent, often a fluorescent non-metal based resonance energy acceptor. For example, by coupling the chelate-lanthanide complex donor to one atom and the acceptor to a second atom, the distance between two atoms can be measured. Generally, the spectral overlap of the donor emission and acceptor absorption is sufficient to enable energy transfer from donor to acceptor as measured by detectable quenching of donor luminescence intensity of lifetime or detectable increase in acceptor luminescence.

Where the atoms are on the same molecule, the methods provide useful information about the structure or conformation of the molecule. For example, the methods are used to monitor the status of a polymerase chain reaction by coupling the donor and acceptor to separated atoms of a diagnostic oligonucleotide. As the concentration of target DNA increases, the percentage of diagnostic oligonucleotides hybridized to target DNA increases which in turn increases the mean distance between the labelled atoms. This increased mean distance is detected as a decrease in energy transfer between the donor and acceptor. Where the atoms are on different molecules, the methods provide useful information on the interactions or relative locations of the two molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Spectra of DNA labelled with either TMR or DTPA-cs124-Tb. Dashed and solid lines are the absorption and emission spectra of TMR, respectively. Solid line with circles is the emission spectrum of DTPA-cs124-Tb on DNA. The TMR emission spectrum shown above was obtained on a steady-state fluorimeter. The spectral overlap between terbium emission and TMR absorption enables energy transfer to take place.

Figure 2A: Emission spectrum of a donor-only labelled dsDNA (solid curve with circles), a donor-acceptor labelled dsDNA (solid curve), and the difference spectra (dashed curve). The DNA length is 10bp in all cases. The donor-only curve and donor-acceptor curves are normalized at 546nm. In the donor-acceptor complex, the ratio of donor-strand DNA to acceptor strand is

slightly greater than 1, although figure 3, curve C, shows that approximately 12% of the donor strand is unhybridized. The signal is collected with a 7.5m sec gate after a 90msec delay. Data collected with a 150msec delay was very similar. The 54:1 signal to background of the donor-acceptor curve at 570nm is calculated by dividing the donor-acceptor signal at 570nm by the donor-only signal at 570nm. The latter is calculated by dividing the donor-only signal at 546nm by 240. Background due to detector noise, direct acceptor fluorescence or photon statistics are not significant. The difference spectra represents the sensitized emission. As expected, the shape is nearly identical to that of a TMR-only labelled DNA fluorescence spectrum.

Figure 2B: Donor lifetime-quenching at 546nm on a donor-only labelled 10mer ssDNA (curve A), a series of partially hybridized donor-acceptor 10mer DNA oligomers (curves B and C), and the sensitized emission signal at 570nm (curve D) corresponding to curve B. To generate curves B and C, a single-stranded donor-only DNA (top curve) was titrated with increasing amounts of acceptor-labelled complement and annealed. The solid line through each curve is a two-exponential (four parameter) fit to the data. The percentages of each component represent their amplitude, e.g. curve B is fit to the equation $y=55\%\exp(-t/331\text{msec}) + 45\%\exp(-t/2123\text{msec})$, indicating 55% donor-acceptor complex, and 45% donor-only complex. r^2 residuals in all cases >0.99 and for donor-quenching curve showed no structure. Sensitized emission curve showed residual structure at >1.5m sec possibly due to a small amount ($\leq 1\%$) of signal arising from unquenched donor species.

Figure 3: Spectra of DNA labelled with either CY-5 or DTPA-cs124-Eu. Dashed and solid lines are the absorption and emission spectra of CY-5, respectively. Solid line with circles is the emission spectrum of DTPA-cs124-Eu on DNA. The small signal at 548nm is due to contaminating terbium. All data shown are at 0.5mM donor strand concentration in 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl, D_2O at 5°C. Decreasing concentration by 2 and 4 fold yielded the same results. Emission spectroscopy was done on a laboratory-built spectrometer utilizing a pulsed Nitrogen laser, photon-counting detector and a multichannel scalar with 2msec time-resolution. The CY-5 emission spectrum shown above was obtained on a steady-state SPEX fluorimeter.

Figure 4A: Emission spectrum of a mixture of donor-strand DNA and acceptor strand in approximately 1:0.6 ratio. The signal is collected with a 7.5m sec gate after a 90msec delay.

Figure 4B: Lifetime data corresponding to figure 3, showing donor-only lifetime of 2.5m sec, a biexponential donor-quenching corresponding to a mixture of donor-only and donor-acceptor complexes, and a largely single exponential sensitized emission signal. The latter signal is insensitive to donor-only or acceptor-only species. Donor-only lifetime on single-stranded and double-stranded DNA differ by less than 5%.

DESCRIPTION OF SPECIFIC EMBODIMENTS

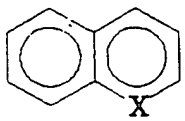
We have synthesized a series of new chemical compounds which bind to lanthanide elements including terbium and europium, and efficiently sensitize them, i.e. allow them to be excited efficiently and subsequently luminesce efficiently. The compounds also enable convenient coupling to macromolecules. We call these compounds lanthanide chelates.

The importance of the invention is several-fold: First, our lanthanide chelates can be used as non-isotopic replacements for radioactive labels. Second, they can be used as alternatives to conventional fluorescent dyes, especially in imaging applications, with the potential for increased contrast. This increased contrast arises because the lanthanide luminescence is extremely long (0.6-2.3 millisecond). If one uses a pulsed excitation source and gated (time-resolved) detection, the intense autofluorescence (background) will decay away, with the labels still emitting. Such autofluorescence currently prevents fluorescent images of many tissue samples. Third, because the chelates are all excited in the same spectral region, two color imaging is possible. Fourth, the lanthanide chelates can be used as extremely efficient donors in luminescent energy transfer analyses. This use enables measurements of distances beyond 100Å, distances currently not measurable with standard fluorescence energy transfer techniques, but of importance in structural biology and medicine.

Our chelates have a number of important advantages: 1. They are easy to synthesize and attach to macromolecules; 2. They are efficiently excited by a nitrogen laser (at 337nm); 3. Some of of them (e.g. DTPA-cs124) can sensitize

both terbium and europium; 4. The lanthanide luminescence from the chelates are extremely intense; for example, the terbium chelate exemplified below luminesces approximately sixty-five times more intensely than DTPA-paraaminosalycilate (DTPA-pAS) when excited at 337 nm; 5. They are chemically stable; 6. They can be used to label nucleic acids and proteins under chemical conditions used in automated synthesizers (e.g. creating phosphoramidites in DNA/RNA technology or protein technology); 7. They are extremely good resonance energy transfer donors. An important element in the success of our chelates in energy-transfer is the fact that there is very little spectral or temporal overlap between the sensitizer's emission and the lanthanide's emission. In contrast, DTPA-pAS has very significant temporal and spectral overlap and provides a poor energy transfer donor; and, 8. They are useful as radiation-hardened fluorophores in X-ray microscopy.

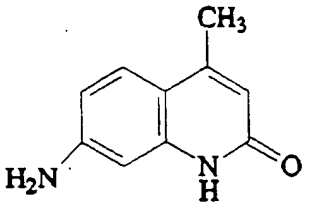
Our lanthanide chelates comprise a polynuclear heterocyclic aromatic sensitizer of the general formula:

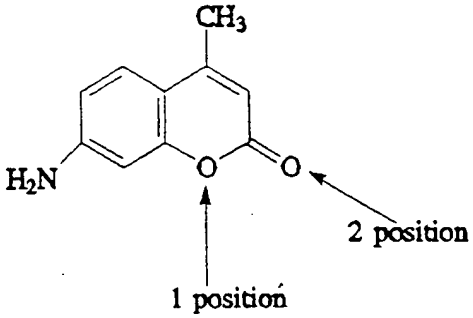


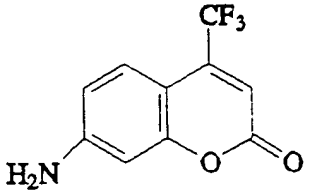
where X comprises an atom from periodic group 5 or 6.

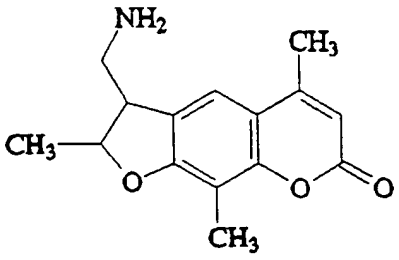
Suitable sensitizers may include a variety of additional structure including structures where the above general formula comprises a structurally minor portion of the sensitizer. Generally, at least one of the position 2-8 carbon atoms, preferably position 2 or 4, is oxidized, preferably to a carbonyl (i.e. double bonded to an oxygen atom). Positions are conventionally numbered: counter-clockwise from the heteroatom which is position number 1. Frequently, another position 2-8 carbon atom, preferably the other 2 or 4 position, is substituted (i.e. a hydrogen atom is replaced) with a group comprising an alkyl, vinyl, oxy (including hydroxyl, alkoxy, carboxyl), carbonyl or substituted nitrogen (e.g. nitro-, amino including substituted amines, etc.), cyano, acetate, etc. group, or a derivative thereof, particularly halide derivatives. Exemplary sensitizers include rhodamine 560, 575 and 590, fluoresceins, 2- or 4-quinolones, 2- or 4- coumarins, or

derivatives thereof e.g. coumarin 445, 450, 490, 500 and 503, 4-trifluoromethylcoumarin (TFC), 7-diethyl-amino-cumarin-3-carbohydrizide, etc., and especially carbostyril 124 (7-amino-4-methyl-2-quinolone), coumarin 120 (7-amino-4-methyl-2-coumarin), coumarin 124 (7-amino-4-(trifluoromethyl)-2-coumarin), aminomethyltrimethylpsoralen:









A wide variety of derivatives of the above general formula may be used as sensitizers, so long as the resultant chelate provides the requisite lanthanide binding and luminescence enhancement. By enhanced luminescence is meant that a solution of the chelate complexed with a lanthanide, when exposed to light at a wavelength, the complexed lanthanide emits light of greater intensity or lifetime

than an identical sample absent the chelate. The enchancement is usually at least 50%, preferably at least 500%, more preferably at least 5000%, most preferably at least 50,000% greater intensity under at least one set of conditions (e.g. specified concentration, solvent system, etc.) e.g. see conditions described herein.

To effectively excite the resident lanthanide, the chelates generally provide absorbtion maxima between 150 and 750 nm, usually between 200 and 650 nm, more usually between 250 and 550 nm, most usually between 300 and 450 nm. Generally, detected emissions are at least 50 nm, usually at least 100 nm, more usually at least 150 nm greater than the incident light. For example, preferred detected emissions for terbium and europium are 492 and 546 nm and 617 and 695 nm, respectively. Extinction coefficients generally exceed 5,000, usually 8,000, more usually 11,000.

The selection of particular chelator-sensitizer combinations is dependent on the intended application. Criteria include the selected lanthanide, sources of potential background fluoresence, quenching agents, the incident light source, etc. Functionally, suitable chelates for a given lanthanide are identified by coupling a candidate sensitizer to a chelator such as DTPA, complexing with the lanthanide, and identifying complexes capable of enhanced lanthanide luminescent. Assay details are described below.

The chelates are capable of forming high affinity complexes with lanthanides, such as terbium or europium. The chelates bind at least one lanthanide, preferably at least one of terbium and europium, with an equilibrium constant of at least 10^6 M^{-1}, preferably at least 10^8 M^{-1}, more preferably at least 10^{10} M^{-1} under at least one set of conditions described herein. A wide variety of structural moieties can be used to provide the requisite lanthanide binding affinity, so long as the resultant chelate provides the requisite luminescence. However, the lanthanide is usually ionically bound by anionic groups such as carboxylate or phosphonate groups.

Frequently, the chelates comprise one or more structurally distinct chelator portions. Typically, these chelator portions comprise a plurality of structurally constrained anionic groups such as carboxylate or phosphonate groups. In a preferred embodiment, these portions are selected from compounds which themselves are capable of functioning as lanthanide chelators with the

aforementioned binding affinities. For example, such chelator portions include EDTA, DTPA, DOTA, NTA, HDTA, etc. and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, etc. The chelate's lanthanide affinity may also be a cooperative (synergistic) result of the interaction of a plurality of functional groups. For example, one or more amino acids moieties, e.g. glycine, of the chelate can be structurally positioned to bind vacant coordination sites of the lanthanide to enhance overall binding affinity.

Where the chelate comprises a structurally distinct chelator portion, it is usually covalently joined to the sensitizer portion, typically through a linking group. Any linking group that is capable of covalently linking the sensitizer with the chelator and does not preclude requisite lanthanide binding and luminescence may be used. Thus, the linking group may comprise a wide variety of structures. Frequently, the linking group comprises a nitrogen, carboxyl, carbonyl or alcohol group or a nitrogen or carboxyl derivative and is covalently joined to a position 2-8 carbon atom in the general formula. The linking group is often covalently joined to one of the position 2-8, frequently position 7, carbon atoms above. Common linking groups are aliphatic and aromatic amines, which may be primary or secondary, carboxyls and sulfhydryls. The chelator and sensitizer are frequently joined through an amide, anhydride, disulfide, thio-urea, thioether, etc. bond.

The chelates may be synthesized in any convenient way. Many of the disclosed sensitizers and chelators sensitizers are commercially available - others are synthesized or modifed from commercial starting materials according to conventional methods. The two may be coupled by any convenient chemistry, though they are most often directly coupled through functional groups as described herein. For example, some of the preferred chelates are based on a reaction between an anhydride (e.g. of diethylenetriaminepentaacetic acid, caDTPA) and a sensitizer (the DTPA acts as the chelator, binding the lanthanide tightly and preventing radiationless deactivation by water, and the organic compound acts as a sensitizer, allowing efficient excitation of the lanthanide). These chelates may be made by a modification of the procedure of Bailey et al (1984) Analyst 109, 1449-1450, where an amine-containing sensitizers replace pAS. The anhydride of DTPA are separately dissolved in an anhydrous organic solvent, typically dry dimethylsulfoxide. The anhydride and selected sensitizer are then mixed and

allowed to react for approximately an hour. The anhydride reacts with the amine on the sensitizer to form a stable amide bond.

Where desired, the chelate may be coupled to an analyte-specific reagent, organic polymer, macromolecule (esp. biomolecules like nucleic acids and proteins), etc in any convenient way. For example, for covalent coupling to a protein (or other amine containing macromolecule), the chelate can be originally formed using the dianhydride of DTPA. After coupling to the sensitizer, the mixture is then added to the amine-containing macromolecule, either in an organic solvent or in an aqueous solvent. The second dianhydride then reacts with the amine(s) on the macromolecule, forming another amide bond. The reactivity of the amine is sufficiently great that this reaction can be done in an aqueous medium (even though water competes for reaction with the anhydride).

Alternatively, a bifunctional linker can be used to couple the chelate and the macromolecule. For example, a suitable linker may comprise both a thiol reactive group (e.g. maleinide, acetyl halide, etc.) and an amine reactive group (e.g. thiourea, isothiocyanate, etc.). For instance, the mono-anhydride of DTPA may be coupled to a protein by reacting with 3-(2-pyridyldithio)propionyl hydrazide (PDPH).

Chelate-lanthanide complexes are useful as detectable labels in a wide variety of applications. Generally, the methods involve contacting a sample portion with a luminescent complex of a chelate and a lanthanide; exposing the sample portion to light at a first wavelength capable of inducing a first electronic transition in the chelate; and detecting, advantageously with a time delay to minimisze the detection of shorter-lived background luminescence, an emission of light from the sample portion at a second wavelength that is longer than the first wavelength and results from a second electronic transition in the chelate. Specific analytes in the sample may be detected by coupling the chelate to a reagent capable of analyte selectively binding.

The methods are adaptable to a wide variety of samples including biological samples and extracts (such as physiological fluids, nucleic acid and/or proteinaceous solutions, microbial cultures, etc.), environmental samples (such as water sources), industrial, especially chemical reagents, products and wastes, etc. The chelates may be free in solution or restrained in a variety of ways. For

example, the chelates may be preferentially partitioned in or on one phase, solid or liquid, such as adsorbed onto a solid surface or membrane or retained within a bead (e.g. latex microspheres). Thus, the methods are useful in conjunction with sorting (e.g. cell sorting), chromatography, electrophoretic, osmotic and centrifugal separations. Heat and organo-stable chelates are selected for applications involving elevated temperature (e.g. distillations, combustions, etc.) and organic extractions.

The first wavelength (that of the incident light) is selected to optimize the ultimate signal-to-noise ratio of the lanthanide emission. Frequently, the incident light is provided in a form to minimize background absorption. Useful sources include lasers (e.g. nitrogen, helium-cadmium, dye lasers, etc.) and arc lamps (e.g. high-pressure, mercury, xenon, quartz, etc.). Nitrogen lasers are particularly preferred because their 337 nm emission frequency is close to a lanthanide absorbtion maximum. Similarly, the second wavelength is selected to optimize signal-to-noise ratio and in view of the available instrumentation.

The subject chelates may be coupled to a wide variety of compounds to create specific labels, probes, diagnostic and/or therapeutic reagents, etc. Examples include biomolecules such as proteins (antibodies, enzymes, receptors, etc.), nucleic acids (RNA, DNA, etc.), bioactive molecules (drugs, toxins, etc.); solid subtrates such as glass or polymeric beads, sheets, fibers, membranes (e.g. nylon, nitrocellulose), slides (e.g. glass, quartz) and probes; etc.

Many of our chelates are particularly amenable to what we term Luminescence Resonance Energy Transfer, or LRET. LRET is a generalized version of Fluorescent Resonance Energy Transfer, or FRET, a widely used technique in polymer science, biochemistry and structural biology. FRET can be used to measure the distances between two points that are labelled with fluorescent dyes and separated by approximately 10-75Å. The technique is valuable because measurements can be made under physiological (or other) conditions with near-Angstrom resolution and with the exquisite sensitivity of fluorescence measurements. FRET relies on a distant-dependent transfer of energy from one fluorescent dye -- the donor -- to another absorbing or fluorescent dye -- the acceptor. The donor and acceptor are site-specifically placed at the two points that one wishes to measure the distance between.

While lanthanides do not fluoresce, the use of our chelates permits them to be efficiently excited. A non-fluorescent quantum transition of the lanthandide can then effect a non-radiative energy transfer to a suitable and appropriately distanced acceptor. To effect transfer, an acceptor absorbtion must overlap a lanthanide emission. The chelate - acceptor pair is selected for optimal overlap: for longer distance measurements, greater overlap is preferred. Since the lanthanides have lifetimes on the order of a millisecond, the signal-to-noise ratio of sensitized emission of the acceptor in LRET is improved by emission detection through time resolution (pulse delay) or phase modulation. Energy transfer can be detected by donor quenching or, preferably acceptor luminescense.

By using luminescent lanthanide chelators as donors (instead of conventional dyes), and conventional fluorescent dyes as acceptors, we have improved the signal to background of LRET by approximately 100-fold. This improvement allows measurements beyond 100Å, a distance currently unmeasurable using small, conventional fluorescent dyes. This distance regime is important in many biological problems. Using lanthanide chelators as donors also makes distance measurements more accurate, because the chelators minimize the uncertainty in the orientation-dependence of energy transfer. We have also demonstrated the first lifetime measurement of the sensitized emission of the acceptor, a LRET measurement which eliminates problems associated with non-specific or incomplete labelling.

A wide variety of acceptors are useful with our chelate donors. Generally, the selected acceptor will have an absorbance maximum at a wavelength between 25 nm and 250 nm longer than that of the donor chelate. Exemplary acceptors include xanthene dyes such as fluoresceins and rhodamines, coumarins, benzimide dyes, phenanthridine dyes, ethidium dyes, acridine dyes, cyanine dyes such as thiazole orange, thiazole blue, Cy5, Cy5.5, Cy3, etc., carbazole dyes, phenoxazine dyes, porphyrin dyes, quinolone dyes, pycobillyc proteins, e.g. allophycocyanin, R-phycoerythrin, B-phycoerythrin, Bodipy dyes, etc. The acceptors generally emit in the visible or infrared ranges.

LRET is particularly useful to obtain structural and kinetic information about macromolecules in solution, in real time. For example, double-end labeled oligonucleotides provide detectable LRET signalling when bound by nucleic acid

binding proteins, e.g. transcription factors. Accordingly, the methods are used to screen for potential therapeutics that alter the structure or interactions of biomolecules; for example, anti-viral agents are sceened for the ability to alter viral transcription factor-induced alterations in nucleic acid conformation.

The general LRET-based method of detecting the distance between a first position and a second position in a portion of a sample involves: exposing a sample portion comprising the donor lanthanide-chelate complex located at the first position and the acceptor located at the second position to light at a first wavelength capable of inducing a first electronic transition in the donor. The spectral overlap of the donor emission and acceptor absorption is sufficient to enable energy transfer from the donor to the acceptor as measured by detectable quenching of donor luminescence intensity or lifetime or detectable increase in acceptor luminescence intensity or lifetime. Then the intensity of a first emission of light from the sample portion at a second wavelength is detected wherein the second wavelength is longer than the first wavelength and results from a second electronic transition in the donor, wherein the intensity of the first emission of light correlates with the distance between the first and second positions. In other words, the closer the positions, the greater the energy transfer and the greater the donor quenching. Alternatively, one can detect the intensity of a second emission of light from sample portion at a third wavelength, wherein the third wavelength is longer than the first wavelength and results from an electronic transition in the acceptor, wherein the intensity of the second emission of light inversely correlates with the distance between the first and second postions of the sample portion. In other words, the closer the positions, the greater the energy transfer and the greater the acceptor luminescense.

This general method has broad application whenever the static or dynamic distance between to positions, e.g. two atoms or molecules, is of interest. In one specific embodiment, the method is used to monitor the status of a polymerase chain reaction. Here, the sample portion comprises a target nucleic acid strand comprising a first strand portion and a diagnostic nucleic acid strand labeled proximal to one end with the acceptor and proximal to the other end with the donor (i.e. comprising a first atom covalently joined to the donor and a second atom covalently joined to the acceptor, the first and second atoms being separated by a

second strand portion). The first and second strand portions are sufficiently complementary to hybridize under annealing conditions, and the second strand portion is of sufficient length to provide a detectable difference in the aggregate energy transfer from the donor to the acceptor when the first and second strand portions are hybridized as compared with the aggregate energy transfer from the donor to the acceptor when the first and second strand portions are not hybridized. The detectable difference is measured as at least one of a detectable quenching of donor luminescence or detectable increase in acceptor luminescence, and the distance between the first and second atoms indicates whether the nucleic acid strands have hybridized. Thus, as the reaction proceeds, the stepwise increase in the amount of target nucleic acid is reflected in a stepwise decrease in energy transfer.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1.

In this example, we exemplify the technique of luminescent resonance energy transfer (LRET) by introducing a luminescent terbium chelate as a donor, and an organic dye, tetramethylrhodamine, as acceptor. The results are consistent with a Förster theory of energy transfer, provided the appropriate parameters are used. The use of lanthanide donors, in general, and this pair, in particular, has many advantages over more conventional FRET pairs which rely solely on organic dyes. The distance at which 50% energy transfer occurs (R_o) is large, 65Å; the donor lifetime is single exponential and long (millisecond), making lifetime measurements facile and accurate; uncertainty in the orientation factor (κ^2) which creates uncertainty in measured distances is minimized by the donor's multiple electronic transitions and long lifetime; the sensitized emission of the acceptor can be measured with little or no interfering background, yielding a $>$ 25 fold improvement in signal to background over standard donor-acceptor pairs. These improvements are expected to make distances greater than 100Å measurable via LRET. We also report measurement of the sensitized emission lifetime, a

measurement which is completely insensitive to total concentration and incomplete labeling.

In FRET a fluorescent donor molecule transfers energy via a non-radiative dipole-dipole interaction to an acceptor molecule (which is usually a fluorescent molecule). FRET is a standard spectroscopic technique for measuring distances in the 10-70Å range. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced, and the acceptor fluorescence is increased, or sensitized(1). FRET is frequently used in both polymer science and structural biology and has recently been used to study macromolecular complexes of DNA, RNA, and proteins (2-4).

Despite these successes, FRET has had a number of serious flaws which has limited its utility. First, the maximum distance which can be measured has been less than optimal for many biological applications. Second the lifetime of commonly used donor fluorophores are short (typically a few nanoseconds) and multiexponential, making lifetime measurements difficult and of limited accuracy. Third, the signal-to-background of the sensitized emission has been low due to interfering fluorescence from the donor and from direct excitation of the acceptor. Fourth, precise distances have been difficult to determine because the efficiency of energy transfer depends not only on the R^{-6} distance between the donor and acceptors, but also on their relative orientation, as expressed by the κ^2 factor. (The efficiency of energy transfer = $1/(1+R^6/R_o^6)$, where R_o is a function of κ^2 :see Appendix).

The luminescent lanthanide elements terbium and europium are attractive FRET donors because they potentially overcome many of these problems. Because lanthanide emission does not arise from a singlet to singlet transition, energy transfer using lanthanide donors is more accurately called luminescence resonance energy transfer (LRET). The lanthanides have primarily been used in diffusion-enhanced FRET (5) and as isomorphous replacements in calcium-binding proteins (6-8). In addition, Mathis has used europium cryptates with the multichromophoric Allophycocanin to achieve an extremely large R_o of 90Å (9). We have recently presented results showing numerous advantages of using a polycarboxylate-chelate of europium as a donor in conjunction with an organic dye such as CY-5 as the acceptor (10). Here we extend these results to the use of terbium as a donor.

As a model system, we covalently attach donor and acceptor to the 5′ ends of a series of double-stranded DNA oligomers of varying length. The use of DNA in such a model system has been previously shown to be valid for energy transfer measurements between organic dyes (11).

Material and Methods

Synthesis of Labeled DNA Oligomers. Complementary DNAs of 10,12,14 bases in length were synthesized using standard phosphoramidite procedures. An amino-group attached via a six-carbon linker (Glen Research) was incorporated at the 5′ end. The acceptor sequence was that used by Clegg et al (11): 5′-CCA-CTC-TAG-G-3′ (10bp); 5′-CCA-CTG-GCT-AGG-3′ (12bp); 5′-CCA-CTG-CTG-CTA-GG-3′ (14bp). The 5-isomer of Tetramethylrhodamine-isothiocyanate (Molecular Probes. T-1480: abbreviation: TMR) was attached via standard procedures and purified by reverse phase HPLC. Extinction coefficients for TMR attached to DNA were determined to be e_{260}= 33m$\underline{M}^{-1}cm^{-1}$ and e_{556}=93m$\underline{M}^{-1}cm^{-1}$. The donor-strand consisted of complementary DNA labeled at the 5' end with a terbium chelate. The chelate is diethylenetriaminepentaacetic acid coupled to a laser dye, carbostyril 124 (DTPA-cs124). Details of the donor chelate synthesis will be presented elsewhere. Unlabelled DNA oligomers were also synthesized.

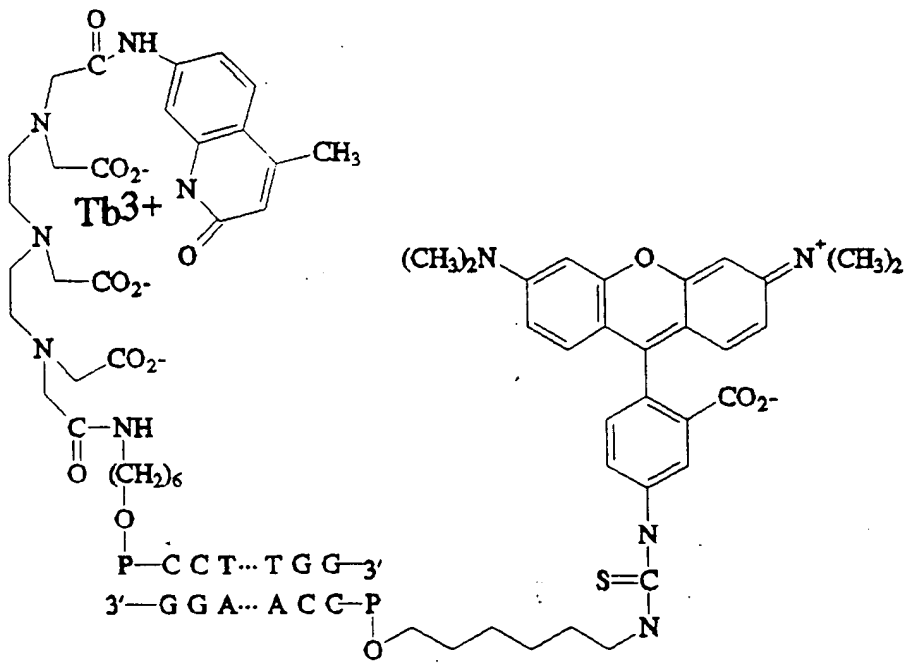
Hybridization Conditions: Donor and acceptor strands were mixed in desired ratio in a D_2O-based buffer containing 10mM Tris, pH 8.0, 10mM $MgCl_2$, 150mM NaCl. Experiments were also performed in an H_2O-based buffer. Donor strand concentration was approximately 200n$\underline{M}$. Oligomers were annealed by heating to 75°C and cooled to the final temperature (22°C or 5°C) over 15 minutes.

Spectroscopy: Absorption measurements were made on a Hewlett Packard 8452A spectrometer. Steady-state fluorescence measurements were on a SPEX Fluorolog fluorimeter. Time-resolved and gated luminescence measurements were made on a laboratory-built spectrometer utilizing right-angle detection with a pulsed Laser-Photonics Nitrogen laser (5nsec pulse width, 40Hz repetition rate), a Gallium-arsenide photon-counting detector, a gated discriminator (Ortec 584) and a multichannel scalar with 2 μsec time-resolution. Polarization studies were also conducted although energy-transfer experiments performed without an analyzer

gave the same results as those using an analyzer. An analyzer was therefore routinely omitted. A temperature-regulated cuvette-holder and a quartz 3mmx3mm cuvette was used. Lifetime data was fit using TableCurve software (Jandel Scientific).

Results and Discussion

The structure of the donor chelate, DTPA-cs124-Tb, and the model system used for energy transfer is shown below:



The donor-chelate has several important features. First, the chelate binds terbium (and europium) extremely tightly — titration with a 100 fold excess of EDTA ($K_b \leq 10^{17}\underline{M}^{-1}$) is unable to displace a measurable amount of terbium. This is in agreement with other DTPA-based chelators (12) and ensures that there is no free terbium. Second, the chelate allows site-specific attachment of terbium to macromolecules. Third, the chelate shields the terbium from non-radiative deexcitation mechanisms, likely resulting in a quantum yield for terbium luminescence near unity in D_2O (see Appendix I). Finally, the covalent attachment

of the laser dye carbostyril 124 overcomes the extremely low absorption cross-section of terbium (< 1 $\underline{M}^{-1}cm^{-1}$) (6). The cs124 absorbs light (ϵ_{328} = 11,000 $\underline{M}^{-1}cm^{-1}$; ϵ_{338} = 8,000 $\underline{M}^{-1}cm^{-1}$) and because of its close proximity to the terbium, transfers energy to the lanthanide (13, 14).

Figure 1 shows the spectral characteristics of the terbium chelate and the tetramethylrhodamine which lead to efficient energy transfer and a large R_o of 65Å in D_2O (60 Å in H_2O). R_o is calculated from standard equations (see appendix). Here we mention two unusual aspects of using a lanthanide chelate as donor: 1) The efficiency of energy transfer can be adjusted, and hence R_o optimized for the particular system being measured, simply by varying the ratio of H_2O to D_2O in the solvent. The H_2O/D_2O ratio affects the efficiency of energy transfer by altering the lanthanide quantum yield (q_D) in our chelate ($q_D \approx 1$ in D_2O; $q_D \approx 0.6$ in H_2O; see Appendix I) (15). 2) The orientation dependence of the energy transfer process is minimized because the terbium has multiple, degenerate, electronic transitions and is therefore an isotropic donor, even if stationary. This minimizes uncertainty in the measured distance due to orientation effects of +/-12% in the worst case(16).

Figure 1 also shows the highly spiked nature of the terbium emission. Donor quenching can be measured without interference from acceptor emission at 492nm and 546nm. Similarly, the sensitized emission of the acceptor can be measured without significant interference from donor luminescence because terbium is nearly silent around 570nm, where TMR is at 70% of its emission maximum. The terbium signal at 570nm is 240x less than at its maximum, 546nm.

When measuring the sensitized emission, we can also eliminate the direct fluorescence of the acceptor by temporal discrimination. We use pulsed excitation, and collect data only after a 90 μsec delay, during which time direct fluorescence of the rhodamine has decayed away. (The acceptor fluorescence, with a lifetime of a few nanoseconds, decays rapidly; we also find a small component — probably either delayed fluorescence or a detector artifact — which decays away within the 90 μsec delay.) The donor, because of its millisecond lifetime, stays excited and capable of transferring energy at the end of the delay period. Consequently, any signal arising around 570nm after the delay is due only to sensitized emission, i.e. fluorescence of the acceptor due to energy transfer.

Figure 2A shows the results of an energy transfer experiment on a partially hybridized 10mer DNA. The *average* energy transfer is 77%. The signal to background of the sensitized emission at 570nm is 54:1. By comparison, the signal to background for sensitized emission when using fluorescein-rhodamine as energy transfer pairs on the same DNA is approximately 1. Because the background is so small in our case, small signals become measurable, and hence distances much greater than R_o are expected to be possible. Horrocks and Bruno, for example, have shown the ability to measure distances of $4R_o$ (R_o=3.1Å) utilizing the dark-background sensitized emission of tyrosine to terbium energy transfer (6).

We can isolate the sensitized emission signal from donor luminescence even in regions where donor luminescence is significant. In a procedure analogous to that used by Clegg et al (11), we can subtract the donor luminescence at all wavelengths, leaving the sensitized emission signal. The efficiency of energy transferred is then simply the area of the corrected sensitized emission, divided by the total corrected area:

$$\text{efficiency of energy transfer} = (f_A/q_A) / (f_A/q_A + f_D) \quad (1)$$

where f_A is the area under the sensitized emission curve, q_A is the fluorescence quantum yield of the acceptor, and f_D is the area under the donor luminescence curve. One can determine the quantum yield of the acceptor by a comparison with the donor quenching data. Based on a quantum yield of 0.174 for TMR (see Appendix II), equation 1 yields an average energy transfer of 77%.

Figure 2B shows the lifetime data on a series of 10mer DNA oligomers. The donor-only (single stranded DNA) signal is single exponential with a lifetime of 2.14msec. (A terbium labeled DNA oligomer hybridized to its complement is single exponential with a lifetime of 2.80m sec. The difference in lifetime between double-stranded and single-stranded terbium-only DNA is likely due to different radiative rates arising from different symmetries surrounding the terbium, rather than different quantum yields.) A titration with increasing amounts of acceptor strand shows bi-exponential donor quenching (curves B and C). The long-lifetime component corresponds to unhybridized, donor-only single stranded DNA; the short component corresponds to those terbium-strands that are hybridized with acceptor strands. As expected, increasing the amount of acceptor strand increases

the amplitude of the short component and decreases the amplitude of the long component, while leaving their lifetimes unchanged (compare curves B and C). That the long-time component equals the donor-only signal is an important internal control which shows that intermolecular energy transfer is not significant. The lifetime of the short component corresponds to an energy transfer in the donor-acceptor complex of 88% (1-331μsec/2809μsec). By comparison, the energy transfer on the same 10mer DNA with the same six-carbon linkers using fluorescein-TMR pair is 23% (11).

In calculating the efficiency of energy transfer based on the sensitized emission curve, we ignore the short time component since this is due to residual signal arising from the direct fluorescence of the acceptor. (No gate was used for this data). Multiple experiments show the long-time component is repeatable to within 10% in the worst case, and usually repeatable within a few percent. The short-time component, however, is highly variable since there is a very large, very short spike due to direct fluorescence which cannot be resolved.

At two-fold excess of acceptor strand, there is still a 10-12% unhybridized component. A similar phenomenon has been seen with dye-labelled oligomers (17) and in FRET experiments with europium substituted in our chelate (10). In our case it does not appear to be a simple melting-temperature phenomenon since it is present at both 5°C and 22°C. The reason for this is under investigation. It is unlikely that this residual unquenched donor signal is due to fundamental lanthanide photophysics since this would require an uncoupled magnetic dipole transition, a situation which is not present since all terbium (and europium) luminescence arises from the same excited state (18, 19).

Figure 2B also shows the lifetime of the sensitized emission at 570nm corresponding to the biexponential donor-quenching (curve B). The sensitized emission decay can be accurately fit to the equation: y=33%exp(-t/45μsec) + 67%exp(-t/326μsec). The 45msec component corresponds to direct fluorescence from the acceptor or a detector artifact (which can be eliminated by gating). The 326μsec component is due to energy transfer on the donor-acceptor complex, and agrees extremely well with the 329-331μsec donor quenching component. Note that after approximately 90 μsec, the only species which contribute to the sensitized emission signal is the donor-acceptor complex — donor-only or acceptor-only do

not contibute. This significantly minimizes the problem of incomplete labeling. The sensitized-emission lifetime signal is also insensitive to total concentration, to quantum yields, and to non-energy-transfer effects which can cause donor quenching.

Table 1 summarizes the lifetime and energy transfer data on donor-acceptor labelled DNA duplexes of 10,12, and 14bp length.

Table I

	Lifetime (μsec)	Efficiency of energy transfer	Calculated Distance (Å)	Clegg *et al.* Distance (Å)
10mer	336	0.88	46.6	55.5
12mer	724	0.74	54.6	56.4
14mer	1154	0.59	61.2	61.0

Data from multiple experiments show donor quenching and sensitized emission lifetimes for a given length DNA agree always within 10%, usually within a few percent. As expected, there is a decrease in energy transfer with increasing distances. For comparison we include the distances determined by Clegg *et al.* using fluorescein-TMR (11). Both results are consistent with the DNA double-helix geometry, although differing salt conditions and donor-lifetimes lead to different dye positions, and hence different measured distances. We have fit our distances using the Clegg *et al.* model of the DNA helix and attached dyes. With only three data points, it is not possible to resolve uniquely all the parameters in the model, but nevertheless, a good fit to their model is achieved if it is assumed that the terbium chelate and/or acceptor are fairly close to the DNA helix. This reduces the modulation seen in their FRET data, which arises because of the helical geometry of the DNA and the fact that their donor and acceptor are extended away from the helix (19Å and 13Å, respectively). This difference is qualitatively reasonable since the long-lifetime of our donor is expected to allow constrained diffusion of the donor and acceptor within the limits placed by the six-

carbon linkers, and because of the greater ionic strength used here, which minimizes charge repulsion.

In summary, the data are consistent with the geometry of the double-helix DNA if the energy transfer data are derived based on the dipole-dipole Förster-type mechanism. Numerous technical advantages of luminescence resonance energy transfer make this a technique well suited for measurements on biologically interesting macromolecules.

Appendix I

Calculation of R_o: R_o, the distance at which 50% of the donor's excited state energy is transferred to the acceptor, is calculated from standard equations (1):

$$R_o = (8.79 \times 10^{-5} J \kappa^2 n^{-4} q_D)^{1/6} \text{ Å} \quad (2)$$

where q_D is the luminescence quantum yield for donor emission in the absence of acceptor, J is the spectral overlap of the donor emission (f_D) and acceptor absorption (e_A) ($J = \int f_D \epsilon_A \lambda^4 d\lambda$), n is the index of refraction and k^2 is a geometric factor related to the relative angles of the two dipoles. Here we evaluate each of the terms in equation 2 and discuss their uncertainty.

The index of refraction, n, varies from 1.33 for water to 1.39 for many organic molecules. We have used 1.33. A numerical integration leads to a J overlap integral of $3.8 \times 10^{15} nm^4 \underline{M}^{-1}$. This is an upper limit for J since the 546nm peak of terbium may arise from magnetic dipole, as well as electric dipole transitions (19), and the former do not significantly transfer energy (18). The fraction of magnetic dipole contribution can be calculated theoretically (20, 21), or the problem avoided by using the 492nm line of terbium, which is known to be solely an electric dipole transition (20).

When using organic dyes in FRET, κ^2 is often a significant source of uncertainty and in the worst case, may vary from 0 to 4 (22). With terbium, however, emission arises from multiple electronic transitions which constrain κ^2: $1/3 < \kappa^2 < 4/3$. In addition, it is likely that the acceptor can undergo rotational motion during the millisecond donor-lifetime. This further constrains κ^2 and we assume $\kappa^2 = 2/3$, corresponding to a random orientation rotating rapidly within the donor lifetime.

The luminescence quantum yield of the terbium, q_D, is difficult to accurately determine because of terbium's intrinsically low absorbance. q_D, however, is likely very close to 1 in D_2O (see below). Note that when calculating R_o, it is important to use the terbium quantum yield ($\approx$ 1 in D_2O), not the quantum yield of the entire chelate. The quantum yield of the entire chelate equals the lanthanide quantum yield times the fraction of energy absorbed by the cs124 that is transferred to the lanthanide.

Quantum yield of lanthanide emission: There are several lines of (indirect) evidence which argue $q_D \approx 1$ in D_2O. First, emission arises from 4f-4f inner shell electrons which are shielded from the solvent and other sources of non-radiative deexcitation by the chelate. The 1.2 H_2O molecules in the primary coordination sphere of the terbium in our chelate (data not shown) are the primary source of non-radiative deexcitation, but these are replaced by D_2O, which do not significantly deactivate terbium (15, 23). The nonwater ligands, carboxylate groups and amine nitrogens are extremely inefficient at deactivating the terbium excited state (23). Via temperature studies (24), we have also looked for quenching effects of the cs124 and have found none.

A second line of evidence supporting $q_D \approx 1$ in D_2O comes from the work of Elbanowski and coworkers who directly measured the quantum yield of a 1:3 mixture of terbium: EDTA in H_2O, and found a value of 0.54 (25). This measurement is difficult and of unknown accuracy, but nevertheless, it suggests a high quantum yield even in H_2O, and the quantum yield in D_2O is expected to be considerably higher. (There are probably 2 water molecules coordinated to the terbium in their complex (23)).

The third line of evidence comes from energy transfer experiments using terbium as a donor in thermolysin (8, 26), and as an acceptor in invertebrate calmodulin (6), where the assumption (sometime implicit) of unity quantum yield in D_2O gives good agreement with x-ray crystallography data.

Appendix II

Calculating the fluorescence quantum yield of the acceptor: By comparing the donor-quenching lifetime data with the areas and using equation 1 it is possible to measure the quantum yield of the acceptor. This is a general and new method

for measuring quantum yields of any dye whose absorption overlaps the emission of terbium (or europium). It has the advantage over more conventional methods of measuring quantum yields in that the measurement involves only one sample — the actual sample of interest — rather than comparing a reference to the sample.

To evaluate the quantum yield of TMR, we assume the unknown in equation 1 is q_A and take the average efficiency of energy transfer to be 77.6%, as determined from curve C (supra). Based on the integrated areas (620 for f_A and 1032 for f_D, in arbitrary units), this yields $q_A = 0.174$. By comparison, free tetramethylrhodamine in phosphate-buffered saline has a quantum yield of 0.25, as measured by standard techniques (27).

Parenthetical references of Example 1.

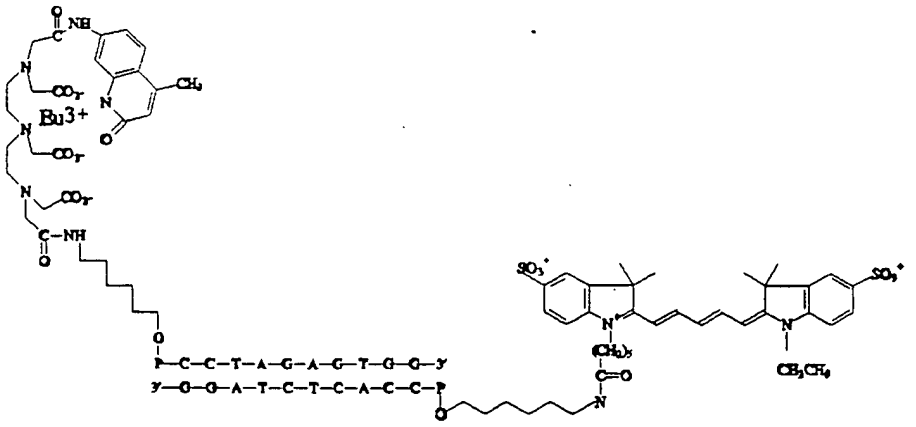
1) Cantor and Schimmel, (1980) *Biophysical Chemistry.* (W. H. Freeman and Co., San Francisco); 2) Clegg, (1992) *Methods Enzymol.* **211**:353-388; 3) Selvin, (1994) *Methods Enzymol.* **246**, (in press); 4) Coker et al., (1994) *Resonance Energy Transfer.* (VCH Publishers, Inc.) (in press); 5) Stryer et al., (1982) *Annu. Rev. of Biophys. and Bioengin.* **11**:203-222; 6) Bruno et al., (1992) *Biochem.* **31**:7016-7026; 7) Cronce and Horrocks, (1992) *Biochem.* **31**:7963-7969; 8) Horrocks, et al., (1975) *Proc. Natl. Acad. Sci. USA* **72**:4764-4768; 9) Mathis, (1993) *Clin. Chem.* **39**:1953-1959; 10) Selvin et al., (1994) *JACS* (in press); 11) Clegg et al., (1993) *Proc. Natl. Acad. Sci., USA* **90**:2994-2998; 12) Oser et al., (1990) *Anal. Biochemistry* **191**:295-301; 13) Abusaleh and Meares, (1984) *Photochem. & Photobiol.* **39**:763-769; 14) Kirk et al., (1993) *J. Phys. Chem.* **97**:10326-10340; 15) Horrocks et al., (1977) *JACS* **99**:2378-2380; 16) Stryer, (1978) *Ann. Rev. Biochem.* **47**:819-46; 17) Cooper and Hagerman, (1990) *Biochemistry* **29**:9261-9268; 18) Dexter, (1953) *J. Chem. Phys.* **21**:836-850; 19) Bunzli, (1989) *Luminescent Probes*, ed. Bunzli, J.-C.G., Choppin, G.R. (Elsevier, New York), pp. 219-293; 20) Carnall et al., (1968) *J. Chem. Phys.* **49**:4412-4423; 21) Gorller-Walrand et al., (1991) *J. Phys. Chem.* **95**:3099-3106; 22) Dale et al., (1979) *Biophys. J.* **26**:161-194; 23) Horrocks and Sudnick, (1979) *J. Am. Chem. Soc.* **101**:334-350; 24) Alpha et al., (1990) *Photochem. and Photobiol.* **52**:299-306; 25) Elbanowski et al., (1989) *Monat. Chem.* **120**:699-703; 26) Berner et al., (1975) *Biochem. & Biophys. Res. Commun.* **66**:763-768; and 27) Waggoner, (1994) *Methods Enzymol.* **246** (in press).

Example 2.

In this example, we exemplify the technique of fluorescence resonance energy transfer (FRET) by introducing a europium chelate as donor and an organic dye, CY-5 as acceptor. The use of lanthanide donors, in general, and this pair, in particular, has many advantages over more conventional FRET pairs which rely solely on organic dyes. The R_o is large, 70Å; the donor lifetime is single exponential and long (2.5msec in D_2O); the orientation factor which creates uncertainty in measured distances is minimized by the donor's multiple electronic transitions and long lifetime; the sensitized emission of the acceptor can be measured with little or no interfering background, yielding a >50 fold improvement in signal to background over standard donor-acceptor pairs. This improvement in signal to background is expected to make distance measurements of greater than 100Å feasible. We also measure the sensitized emission lifetime, a measurement which is independent of total concentration and incomplete labeling.

We have used a luminescent europium chelate as donor and an organic dye, CY-5 as acceptor. This luminescence resonance energy transfer (LRET) has several advantages over the more conventional FRET[2]. The distance at which 50% of the energy is transferred (R_o) is large, 70Å; the donor lifetime is single exponential and long (0.63msec in H_2O; 2.5msec in D_2O), making lifetime measurements facile and highly accurate; the orientation dependence (κ^2) of energy transfer is minimized by the donor's multiple electronic transitions and long lifetime, limiting uncertainty in the measured distance due to orientation effects to +/-12% in the worst case[3]; the sensitized emission of the acceptor can be measured with little or no interfering background, yielding a >50 fold improvement in signal to background over standard donor-acceptor pairs and enabling distances several times R_o to be measured[4]. We also measure the sensitized emission lifetime which, in our case, is independent of total concentration and incomplete labeling.

We have used both terbium[5] and europium as donors, and the results for europium are presented here. A schematic diagram of the donor-acceptor model system comprising double stranded DNA with europium chelate (donor) at one 5′ end and CY-5 at other 5′ end is shown below:



The europium chelate, (diethylenetriaminepentacetic acid-carbostyril 124-Eu: trivial name, DTPA-cs124-Eu) was made by a modification of the procedure of Bailey[12], starting with the dianhydride of DTPA (Sigma), carbostyril 124 (Aldrich), and the synthetic DNA base-protected and on the column to ensure that labelling occurs only at the 5' amino group. The cs124 effectively increases the absorption cross section of the europium to approximately 8000 $\underline{M}^{-1}cm^{-1}$ at 337nm, where we excited the donor with a pulsed Nitrogen laser. A 100-fold excess of EDTA did not remove any noticeable amount of europium from the DTPA-cs124 chelator. The acceptor was 5' labeled with CY-5[13] (Biological Detection Systems) via standard methods. Unlabelled complementary DNA oligomers were made as controls. All DNA was reversed-phase HPLC purified. In this system, the double-stranded DNA oligomer serves as a rigid tether to establish a defined distance between the europium donor and the CY5 acceptor. The points of attachment of the donor and acceptor are separated by 42Å, although the dye positions retain limited variability due to the flexible six-carbon linkers used for attachment [6,7].

Figure 3 shows the spectral characteristics which lead to the unusually large R_o of 70Å in D_2O (56Å in H_2O). R_o is determined from standard equations[1] based on a calculated spectral overlap (J) of 6.55 $x10^{15}$ $\underline{M}^{-1}nm^4$, an orientation factor (κ^2) of 2/3, an index of refraction of 1.33, and a quantum yield for europium luminescence in D_2O of one (0.25 in H_2O)[8]. In calculating R_o it is important to use the quantum yield of the lanthanide emission, and not the quantum yield of the entire chelate, and to include in the spectral overlap (J) calculation only those

transitions which are electric dipole. The europium emission at 617nm, which is used here for energy transfer, has been shown to be "forced" electric dipole[9], and hence Förster's theory of energy transfer is applicable. The europium emission at 596nm cannot couple to an acceptor because it is a magnetic dipole transition and so is not included in the spectral overlap calculation[10].

We can measure the sensitized emission of the acceptor without significant interference from either donor emission or direct acceptor fluorescence. At 668nm, europium is nearly silent (europium emission at 668nm is 125 times less than at its 617nm maximum) and by using pulsed-excitation and gating off the detector for 90msec, the direct fluorescence of the carbostyril sensitizer in the donor complex and the direct fluorescence of the CY-5 are completely eliminated, while the europium stays excited and capable of energy transfer[11].

Figure 4A shows such a dark-background sensitized emission experiment. Here the ratio of donor to acceptor strands is approximately 1:0.6; we intentionally add less acceptor than donor to show the ability of our system to analyze heterogeneous signals. The *average* fraction of energy transfer in figure 4A is 57%. The signal at 668 nm arises from sensitized emission of CY-5, i.e. fluorescence due only to energy transfer. We calculate the signal/background at 668 nm to be 94:1 (where background is due to a small amount of europium luminescence), a factor of 50-100 improvement in signal/background over the sensitized emission signal from fluorescein-rhodamine, one of the best donor-acceptor pairs, attached to the same 10mer.

Figure 4B shows lifetime data corresponding to Figure 4A. The donor-only signal is single exponential with a lifetime of 2.52msec. The donor quenching signal fits a biexponent extremely well (r^2=0.998): y = 63%exp(-t/0.22msec) + 37%exp(-t/2.40msec). The long-time component corresponds to the donor-only species. That the long-time component nearly equals the donor-only lifetime is an internal control which shows that intermolecular energy transfer is at most 5%. The short time component arises from intramolecular energy transfer in the hybridized donor-acceptor complex and corresponds to 91% quenching (1-0.22msec/2.52msec), and a donor-acceptor distance of 46Å. (Fluorescein-rhodamine on the same DNA with C-6 linkers yields 22% energy transfer[7].) A titration with increasing acceptor concentration increases the fraction of the short

time-component but does not change its lifetime, as expected. At two-fold excess of acceptor strand, a 10% component corresponding to donor-only signal remains, presumably due to unhybridized donor strands.

Figure 4B also shows the lifetime of the sensitized emission. The sensitized emission lifetime signal is fit to a biexponential (r^2=0.999): y = 40%exp(-t/59μsec) + 60%exp(-t/0.25msec). The short-time component is due to direct fluorescence of the acceptor and can be eliminated by gating the detector. The 0.25msec component is due to an energy transfer of 90% (1-0.25/2.52msec), in excellent agreement with the short-time component of the donor quenching. A very small long-time component ($\approx$1%) can be seen due to direct donor fluorescence.

In summary, luminescence energy transfer yields results consistent with a Förster theory assuming the appropriate parameters are used. Based on the large R_o, the ease and reproducibility of our lifetime measurements, and the excellent signal to background, distances significantly greater than 100Å are measurable.

FOOTNOTED REFERENCES IN EXAMPLE 2.

(1) Cantor and Schimmel, (1980) *Biophysical Chemistry*, W. H. Freeman and Co., San Francisco, Vol. 2; (2) Lanthanides in energy transfer have been used in diffusion enhanced FRET (Stryer, L., Thomas, D. D., Meares, C. F. (1982) In *Annual Review of Biophysics and Bioengineering*, L. J. Mullins, Ed., Annual Reviews, Inc., Palo Alto, CA, **11**:203-222). They have also used been used with multichromophoric Allophycocanin (Mathis, (1993) *Clin. Chem.* **39**:1953) and as isomorphous replacements in calcium-binding proteins (Horrocks et al., *Proc. Nat. Acad. Sci. USA* (1975), **72**:4764; Cronce and Horrocks, (1992) *Biochem.* **31**:7963); (3) Stryer, (1978) *Ann.Rev. Biochem.* **47**:819-846; (4) The ability to measure energy transfer beyond R_o using sensitized emission has been shown by Bruno and Horrocks. They used terbium as the acceptor and tyrosine as the donor. With an R_o of 3Å they measured out to 12Å. (Bruno et al., (1992) *Biochem.* **31**:7016); (5) Selvin and Hearst, (1994) *Proc. Natl. Acad. Sci, USA* (submitted), (6) Cardullo et al., (1988) *Proc. Natl. Acad. Sci., USA* **85**:8780; (7) Clegg et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**:2994; (8) The exact quantum yield is difficult to determine although the long-lifetime and lack of radiationless deactivation mechanisms make it likely that the quantum yield is close to one in D_2O. This

assumption has given distances in agreement with x-ray crystallography studies (See ref 4). In H_2O, the quantum yield is decreased because there are 1.3 water molecules in the primary coordination sphere of the lanthanide in our chelate (Horrocks and Sudnick, (1979) *J. Am. Chem. Soc.* **101**:334); (9) Bunzli, J.-C.G. (1989) In *Lanthanide Probes in Life, Chemical and Earth Sciences, Theory and Practice Luminescent Probes*; Bunzli, J.-C.G. & Choppin, G.R. Ed., Elsevier, New York, pp. 219-293; (10) Dexter, (1953) *J. Chem. Phys.* **21**:836; (11) Morrison has used gated integration to increase the signal to background of the sensitized emission with organic dyes. Morrison, (1988) *Anal. Biochem.* **174**:101; (12) Bailey et al., (1984) *Analyst* **109**:1449; and (13) Mujumdar et al., (1993) *Bioconj. Chem.* 4:105.

Example 3. Terbium chelate -fluorescein energy transfer.

This example employed a terbium chelate (Terbium-diethylenetriamine-pentaacetic acid coupled to carbostyril 124) transferring energy to fluorescein. The donor and acceptor are separated by an 8 mer DNA duplex oligonucleotide modified with a primary amine on the 5′ end. The acceptor is attached to the 5′ end of a complementary oligonucleotide. The sensitized emission is measured with no background at around 520 nm. Furthermore, there is excellent overlap between 492 nm donor emission line and fluorescein absorbance, leading to a large R_o. By using a pulsed excitation source and monitoring at 520 nm, any signal arises only from sensitized emission. The 8-mer oligonucleotide is used to rigidly separate the donor and acceptor, and the complex is immersed in a viscous sucrose solution to eliminate intermolecular interactions, though the sucrose solution is generally not necessary when the oligonucleotide concentration is maintained below about 0.25 uM. Sensitized emission signal to background ratio at 520 nm is approximately 400:1. The extent of energy transfer based on both donor intensity quenching and the integrated sensitized emission area is 70%. Measurements of the unquenched donor lifetime (1.5 msec) in the absence of acceptor, and the sensitized emission lifetime in the donor-acceptor complex (lifetime 250 usec), indicate a quenching of 85%. The difference between the 70 and 85% is due to the small fraction of unhybridized donor labeled DNA which cannot transfer energy.

Example 4. Assay for Enzymatic (Proteolytic) Activity

Fluorescence resonance energy transfer (FRET) has been used to study enzymatic activity where the distance between two labeled points changes as a function of this activity. In general, where FRET has been used, LRET can also be used, often with significant improvements. For example, Matayoshi et al. (1990) Science 247:954-958, used FRET to measure HIV protease activity by labeling a substrate peptide with a donor and acceptor on either side of a protease binding/cutting site. Because of the close proximity of the donor and acceptor, the donor fluorescence in the intact peptide was highly quenched. Upon addition of a protease, the peptide was cleaved, the donor and acceptor diffuse away from each other and the donor fluorescence increased approximately forty fold. The sensitivity of the assay is largely determined by the extent of donor quenching in the intact peptide. The lack of 100% quenching causes background fluorescence even in the absence of proteolytic activity. The authors note that they used a donor with a relatively long lifetime (tens of nanoseconds) because this helps increase donor quenching of the intact peptide. (The reason is that the peptide is flexible and a long-lifetime allows the peptide time to flex such that the donor and acceptor come close together, resulting in donor quenching.)

Replacing a standard fluorescent donor with a lanthanide chelate provides significantly reduced background, and hence, better sensitivity. The lanthanide chelate has a lifetime which is $> 10^5$ times longer than organic fluorophore, which leads to greater donor quenching due to the peptide flexibility. In addition, any residual donor luminescence can be discriminated against by using pulsed excitation and turning of the detector for some fraction of a millisecond after the pulse. If the donor is quenched by 90% in the intact peptide, for example, the remaining 10% luminescence will have a lifetime 1/10 of the unquenched chelate, or approximately 100 usec. By gating off the detector for a few hundred microseconds, this residual donor luminescence is prevented from reaching the detector, while the unquenched luminescence which arises after proteolysis, which has approximately a millisecond lifetime, is relatively unaffected by the gating. Lastly, the exceptional efficiency of energy transfer from the lanthanide chelates to acceptors allows the attachment points of the donor and acceptor to be placed

farther apart on the peptide than is possible with standard dyes. This is an important consideration when the binding site of the enzyme is large.

Example 5. Detection of genes and DNA sequences

FRET has been used to detect specific DNA sequences, Morrison et al. (1989) Analytical Biochemistry 183:231-244, and Lee et al. (1993) Nucleic Acids Research 21:3761-3766. The primary advantage of using FRET in this application is that the technique is potentially sensitive (i.e. capable of detecting one or a few "target" DNA sequences), and can be done in a homogenous format. The same technique described by Lee et al. is now a commercially available product from Applied Biosystems known as "Taq-Man". In this application, a DNA probe complementary to the target DNA sequence is made with donor and acceptor attached. When the probe is intact and single-stranded, the donor is highly quenched because the acceptor is in close proximity. When the probe hybridizes to the target DNA, the probe is degraded by a specific endo or exonuclease. The donor is then free to diffuse away from the acceptor molecule, and donor fluorescence increases. If there is no target DNA, no specific degradation occurs and no specific increase in donor fluorescence occurs.

This system can also exploit amplification: there are many probe DNA molecules and each time one binds, the probe in the double stranded product is hydrolyzed by the nuclease, and fluorescence increases. As soon as one probe molecule is degraded, another probe molecule can bind and the enzyme repeats the process. Hence, for only one target, the fluorescence from many probe molecules can be generated. A variety of exonucleases and endonucleases are compatible with amplification; for example, Applied Biosystems utilizes Taq-polymerase, which has a 5′ exonuclease activity. If the probe comprises RNA, RNAaseH may be used to exploit amplification.

In any of these FRET-based systems for DNA detection, replacing the organic dye donor with a lanthanide chelate improves the assay. The extent of donor quenching in the intact single-stranded probe is considerably greater because of the lanthanide's long-lifetime and efficient energy transfer. Any residual donor luminescence can be discriminated against with time-gating. The result is

significantly decreased background (exactly analogous to the protease experiment outlined above).

Example 6. Lifetime-tailored Dyes

As discussed above, the lanthanides can be used as luminescent labels in microscopy and detection in general. By using pulsed excitation and time-delayed detection, the lanthanide's long excited-state lifetime (milliseconds) enables signal to be isolated from background fluorescence, which tends to be of nanosecond duration. The long-lifetime, however, has the drawback that the signal intensity per molecule is necessary weak: a molecule with an excited state lifetime of 1 msec can emit at most 1,000 photons/sec. This is particularly significant at high illumination intensities, where the slow-emitting molecule may saturate. At low illumination intensity, the long lifetime is not deleterious because any one lanthanide is being excited at a rate less than the inverse of the lifetime. Ideally, one would have a luminescent label in which the lifetime can be tailored to the optimal time-scale - long enough that background can be discriminated against, short enough that signal intensity is not significantly compromised. Since the background tends to have nanosecond lifetime, a probe with a microsecond lifetime, for example, would still have a thousand-times longer lifetime, enabling time-discrimination of the signal, but would potentially be able to emit 1,000 times more photons/sec than the millisecond lanthanide. Such lifetime-tailored dyes can be made by utilizing luminescence energy transfer between a lanthanide chelate (as donor) and a fluorescent acceptor. The signal will be the emission of the acceptor due to energy transfer. The lifetime of this emission (τ) will equal the lifetime of the quenched donor and will be:

$$\tau = \tau_o (1- E) \quad (1)$$

where τ_o is the lifetime of the lanthanide chelate in the absence of acceptor and E is the fraction of energy transferred (also called the efficiency of energy transferred). The distance between the donor and acceptor (R) to achieve a given τ can easily be calculated using the standard formula for FRET, Cantor et al. (1980) Biophysical Chemistry, WH Freeman and Co, SF, a formula which has been shown to be applicable to LRET (Selvin et al. (1994) PNAS USA 91:10024-10028;

Selvin et al. *J. Amer. Chem. Soc.* 116:6029-6030; Selvin et al. (1994) *Methods in Enzymology*, K Sauer, ed., Academic Press, Orlando):

$$E = 1/[1+(R/R_o)^6] \quad (2)$$

where Ro can be calculated from the spectral properties of the donor and acceptor. Combining equations 1 and 2 yields the lifetime as a function of distance between donor and acceptor:

$$\tau = \tau_o /[1+(R_o/R)^6] \quad (3)$$

If τ_o is 1.5 msec, then with 90% energy transfer, the sensitized emission will decay with a lifetime of 150 usec. With 99% energy transfer, the lifetime is 15 usec. If a luminescent label with a lifetime of 15 usec is desired, then the donor and acceptor should be placed such that 99% energy transfer occurs. This corresponds to a distance of 0.465 x R_o. For Tb-chelate as donor, and tetramethylrhodamine as acceptor (Selvin, et al. 1995, supra), where R_o = 60Å, this corresponds to 28Å. A probe with a 1.5 usec lifetime can be achieved by placing donor and acceptor 0.316 R_o apart for 99.9% energy transfer, or 19Å for Tb-chelate and tetramethylrhodamine.

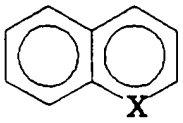
Note that lifetime-tailored dyes can be made to emit at many different colors. Any acceptor which can take energy from the luminescent lanthanide chelate are possible. The acceptor absorption need not overlap with the main emission line of the lanthanide; overlap with less intense emission lines can still yield very efficient energy transfer so long as the acceptor is placed sufficiently close. A donor/acceptor pair with an R_o of only 30Å will still give 99.9% energy transfer at a distance of 9.5Å. Further note that efficient energy transfer can arise even if the donor lanthanide has a relatively poor quantum yield for emission. While Tb and Eu quantum yields tend to be fairly high in the appropriate chelate, other lanthanides and transition elements with significantly lower quantum yields, such as Pr, Nd, Sm, Dy, Ho, Er, Tm and Ru, Os, and Sm become useful donors as well. Finally, note that because the efficiency of energy transfer will in general be high (>90%) almost every photon which would have been emitted from the lanthanide, now gets emitted by the acceptor, but in a shorter period of time. The only significant loss in photons arises from the non-unity quantum yield of the acceptor. This can be minimized by choosing acceptors with high quantum yields.

A wide variety of different linker molecules may be used to create these lifetime-tailored dyes. Generally, the subject linkers provide separation distances from about 4 to 60, preferably from about 9 to about 30Å. The composition of the linker is selected based on the needs of the application, e.g. hydrophobic polymeric linkers may be used to provide membrane permeable dyes. Peptides are readily synthesized to useful lengths, and their N- and C-termini can be used to attach the donor and acceptor. By introducing an internal aminophenylalanine (or a lysine), a reactive isothiocyanate is generated for attachment of the complex to a biological (or other) macromolecule. Peptides also have the advantage that the properties of the peptide can be optimized for a particular application; for example, polyproline is very rigid and hydrophobic; polylysine is hydrophilic and highly positively charged; while polyglutamate or polyaspartate are hydrophilic and negatively charged. Other linkers include polymers such as polysaccharides, which have excellent solubility and reactivity, polyimides, and nucleic acids. The polymers may be derivitized to provide improved attachment sites, e.g. a primary amine can be inserted into a nucleic acid strand and converted to an isothiocyanate group for attachment to macromolecules.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A lanthanide chelate comprising a lanthanide chelator covalently joined to a sensitizer, wherein said chelate is capable of binding a lanthanide with an equilibrium constant of at least 10^6 M^{-1}, a complex of said chelate and said lanthanide is capable of enhanced lanthanide luminescence, and said sensitizer comprises a polynuclear heterocyclic aromatic compound of the general formula:



wherein X comprises an atom from periodic group 5 or 6.

2. A chelate according to claim 1, wherein a first position 2-8 carbon atom of said sensitizer is substituted with an oxygen atom through a double covalent bond, and a second position 2-8 carbon atom of said sensitizer, different than said first position 2-8 carbon atom, is substituted with a linking group through which said sensitizer is covalently joined to said chelator.

3. A chelate according to claim 2, wherein said first position 2-8 carbon atom is the position 2 or 4 carbon atom and said second carbon atom is the position 7 carbon atom.

4 A chelate according to claim 2, wherein a third position 2-8 carbon atom of said sensitizer, different from said first and second position 2-8 carbon atoms, is substituted.

5. A chelate according to claim 4, wherein said third position 2-8 carbon atom is the position 4 carbon and is substituted with a hydrocarbon or halogen substituted hydrocarbon.

6. A chelate according to claim 1, wherein said sensitizer comprises a 2- or 4-quinolone or a 2- or 4- coumarin.

7. A chelate according to claim 1, wherein said sensitizer comprises carbostyril 124 (7-amino-4-methyl-2-quinolone), coumarin 120 (7-amino-4-methyl-2-coumarin), coumarin 124 (7-amino-4-(trifluoromethyl)-2- coumarin), aminomethyltrimethylpsoralen.

8. A chelate according to claim 1, wherein said chelator comprises DTPA.

9. A method of detecting an analyte which may be present in a portion of a sample by luminescence, said method comprising the steps of :

contacting a sample portion with a luminescent complex of a lanthanide chelate according to claim 1 and a lanthanide capable of binding said chelate with an equilibrium constant of at least 10^6 M^{-1}, wherein said chelate is covalently joined to a reagent capable of selectively binding said analyte;

incubating said sample portion under conditions wherein said reagent is capable of selectively binding said analyte;

exposing said sample portion to light at a first wavelength capable of inducing a first electronic transition in said chelate;

detecting an emission of light from said sample portion at a second wavelength, wherein said second wavelength is longer than said first wavelength and results from a second electronic transition in said chelate;

wherein the detection of said emission of light correlates with the presence of said analyte sample portion.

10. A method of detecting the distance between a first position and a second position in a portion of a sample by resonance energy transfer using a luminescent lanthanide chelate donor and an organic resonance energy acceptor, said method comprising the steps of:

exposing a sample portion comprising said donor located at said first position and said acceptor located at said second position to light at a first wavelength capable of inducing a first electronic transition in said donor, wherein said donor comprises a complex of a lanthanide chelate according to claim 1 and a lanthanide capable of binding said chelate with an equilibrium constant of at least 10^6 M^{-1}, and wherein the spectral overlap of the donor emission and acceptor

absorption is sufficient to enable energy transfer from said donor to said acceptor as measured by detectable quenching of donor luminescence intensity or lifetime or detectable increase in acceptor luminescence intensity or lifetime;

detecting at least one of:

the intensity of a first emission of light from said sample portion at a second wavelength, wherein said second wavelength is longer than said first wavelength and results from a second electronic transition in said donor, wherein the intensity of said first emission of light inversely correlates with the distance between said first and second positions of said sample portion; and

the intensity of a second emission of light from said sample portion at a third wavelength, wherein said third wavelength is longer than said first wavelength and results from an electronic transition in said acceptor, wherein the intensity of said second emission of light correlates with the distance between said first and second postions of said sample portion.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08319

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53
US CL :435/7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANALYTICAL BIOCHEMISTRY, Volume 210, issued 1993, Clark el al., "A Study of Sensitized Lanthanide Luminescence in an Engineered Calcium-Binding Protein", pages 1-6, see especially the abstract and "RESULTS AND DISCUSSION" on pages 2-5.	1-10
Y	US, A, 4,637,988 (HINSHAW ET AL.) 20 January 1987, see especially the abstract, Examples 1-5 in columns 11-13, and the Agents in columns 23-28.	1-10
Y	US, A, 4,837,169 (TONER) 06 June 1989, see especially the abstract and examples 18 and 19 in columns 31-34.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 OCTOBER 1995	15 NOV 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

Int. ..tional application No.
PCT/US95/08319

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochimca et Biophysica Acta, Volume 1102, issued 1992, Ando et al., "Synthesis of a highly luminescent terbium chelate and its application to actin", pages 186-194, see especially the abstract and Results and Discussion on pages 189-194.	1-10
Y	ANALYST, Volume 109, issued November 1984, Bailey et al., "Terbium Chelate for Use as a Label in Fluorescent Immunoassays", pages 1449-1450, see especially the abstract and Figure 3 on page 1450.	1-10
Y	ANALYST, Volume 114, issued November 1989, Canfi et al., "Fluorescent Terbium Chelates Derived From Diethylenetriaminepentaacetic acid and Heterocyclic Compounds", pages 1405-1406, see especially the abstract.	1-8
Y	ANALYST, Volume 117, issued December 1992, Diamandis, "Europium and Terbium Chelators as Candidate Substrates for Enzyme-labelled Time-Resolved Fluorimetric Immunoassays", pages 1879-1884, see especially the abstract and Figure 1 on page 1880.	1-10
Y	ANALYST, Volume 114, issued July 1989, Saavedra et al., "Time-resolved Fluoremetric Detection of Terbium-labelled Deoxynucleic Acid Separated by Gel Electrophoresis", pages 835-838, see the entire disclosure.	1-10
Y	Journal of the American Chemical Society, Volume 115, Number 23, issued 1993, Saha et al., "Time-Resolved Fluorescence of a New Europium Chelate Complex: Demonstration of Highly Sensitive Detection of Protein and DNA Samples", pages 11032-11033, see the entire disclosure.	1-10
Y	US, A, 5,252,462 (DREVIN ET AL.) 12 October 1993, see the entire disclosure.	1-10
Y	US, A, 4,421,654 (PLUEDDEMANN) 20 December 1983, see the entire disclosure.	1-10
X --- Y	US, A, 4,801,722 (HINSHAW ET AL.) 31 January 1989, see especially the abstract and column 6, line 22, through column 11, line 61.	1, 2, 4-6, 9 --- 3, 7, 8, 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08319

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 4,794,191 (HINSHAW ET AL.) 27 December 1988, see especially the abstract and column 6, line 16, through column 11, line 62.	1, 2, 4-6, 9 --- 3, 7, 8, 10
Y	WO, A, 89/10975 (PHARMACIA AB) 16 November 1989, see the entire disclosure.	1-10
X --- Y	US, A, 5,021,567 (JOHNSON ET AL.) 04 June 1991, see especially the abstract, columns 11-19, and claims 11-13.	1, 2, 9 --- 3-6, 8, 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/08319

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S

435/6.7.1.7.2.810; 436/501.63.91.93.96; 530/387 1. 536/23.1.24.1.24.3.24.31.24.32.24.33; 546/152-154.156; 935/77.78

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS, BIOSIS, MEDLINE, WPI, BIOTECH ABS.
search terms: chelate?, quinolin?, quinolon?, lanthanide?, immunoassay?, analyte?, conjugate?